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Mapping and Functional Role of Phosphorylation Sites in the Thyroid Transcription Factor-1 (TTF-1)*

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The phosphorylation of thyroid transcription factor-1 (TTF-1), a homeodomain-containing transcription factor that is required for thyroid-specific expression of the thyroglobulin and thyroperoxidase gene promoters, has been studied. Phosphorylation occurs on a maximum of seven serine residues that are distributed in three tryptic peptides. Mutant derivatives of TTF-1, with alanine residues replacing the serines in the phosphorylation sites, have been constructed and used to assess the functional relevance of TTF-1 phosphorylation. The DNA binding activity of TTF-1 appears to be phosphorylation-independent, as indicated also by the performance of TTF-1 purified from an overexpressing *Escherichia coli* strain. Transcriptional activation by TTF-1 could require phosphorylation only in specific cell types since in a co-transfection assay in heterologous cells both wild-type and mutant proteins show a similar transcriptional activity.

The thyroid transcription factor-1 (TTF-1)[1] is a homeodomain-containing transcription factor (1) that binds to the promoters of thyroglobulin (Tg) and thyroperoxidase (TPO) genes, whose expression is restricted to the thyroid follicular cells (2, 3). Transactivation studies demonstrated that TTF-1 is able to activate transcription from co-transfected thyroglobulin and thyroperoxidase promoters in non-thyroid cells, suggesting that TTF-1 may play an important role in the transcriptional activation of thyroid-specific genes during development (4, 5). However, the presence of TTF-1 protein does not always correlate with active transcription of Tg and TPO genes, as TTF-1 has been demonstrated in tissues other than thyroid, where no Tg and TPO mRNA could be detected. Furthermore, TTF-1 protein has been detected very early during thyroid development, 5 days before the appearance of Tg and TPO mRNAs (6). These data indicate that, in physiological conditions, TTF-1 is not sufficient to activate transcription of thyroid-specific genes. Such a notion is strongly supported by the observation that transgenic mouse lines carrying a thyroglobulin promoter fused to a chloramphenicol acetyltransferase gene express the reporter only in thyroid, again indicating that TTF-1 present in other tissues is unable to activate the Tg promoter (7). Interestingly, the promoters of the surfactant protein B and A genes, exclusively expressed in lung, have been demonstrated to depend on TTF-1 for expression (8, 9). Taken together, these data suggest that the activity of TTF-1 is differentially regulated.

Phosphorylation is perhaps the most frequent post-translational modification of those proteins whose activity is regulated in response to changes in metabolic activity, environmental conditions, and hormonal signals. Many transcription factors are regulated by phosphorylation through several distinct mechanisms (10–13) that can affect either their DNA binding or their transcriptional activity. We have previously demonstrated that, in the rat thyroid cell line FRTL-5, TTF-1 is phosphorylated (14). Furthermore, in a Ha-*ras*-transformed FRTL-5 cells TTF-1 has been demonstrated to be underphosphorylated and unable to activate transcription, suggesting that phosphorylation could be an important mechanism in controlling TTF-1 activity (14). It has also been proposed that Ki-*ras* reduces the capacity of TTF-1 to bind to DNA via a phosphorylation-dependent mechanism (15). We report in this study the mapping of TTF-1 phosphorylation sites. TTF-1 mutants unable to be phosphorylated show normal levels of DNA binding and transcriptional activity in heterologous cells, suggesting that phosphorylation of TTF-1 may have an important role only in specific cell types.

MATERIALS AND METHODS

Plasmids Construction—Two primers (start) CCCGGGAAGCTTCTCCACTCAAGCCAATTAAGGCGG and (end) GCGCGCTCTAGAGAGCAGCGGGCGAATGGTGG were used to specifically amplify the entire coding region of TTF-1 by polymerase chain reaction. *Hin*dIII and *Xba*I sites, respectively, were included in these primers to facilitate cloning. Specific serine codons were changed to alanine codons by polymerase chain reaction as described previously, using primers containing the specific mutations, together with (start) and (end) primers.

The amplified products were cloned into the eukaryotic expression vector Rc/CMV (Invitrogen). Finally, restriction fragments containing the serine-alanine substitutions were excised and subcloned in CMV/TTF-1, D14, or D26, described in De Felice *et al.* (5), to generate the mutated constructs indicated in Figs. 2 and 4.

Cell Culture and Transfection Assay—The FRTL-5 cell line has been previously described in detail (16). Briefly, FRTL-5 cells were grown in Coon's modified F-12 medium (Seromed) supplemented with 5% calf serum (Life Technologies, Inc.) and six growth factors as described by Ambesi-Impiombato and Coon (16). HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf medium (Life Technologies, Inc.). For transient expression assays, before the *in vivo* labeling, cells were plated at 8×10^5/100-mm diameter tissue culture dish 1 day prior to transfection. Transfections were

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[1] The abbreviations used are: TTF-1, thyroid transcription factor-1; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis.

carried out by the calcium phosphate co-precipitation technique as described elsewhere (4, 17). For transactivation experiments, cells were plated at 4×10^5 cells/60-mm diameter tissue culture dish 5–8 h prior to transfection. One microgram of a plasmid containing the luciferase gene under the control of the cytomegalovirus enhancer/promoter was used to monitor for transfection efficiency. Cell extracts were prepared 48 h after transfection, and the chloramphenicol acetyltransferase and luciferase activities were determined as described previously (18, 19).

Biosynthetic Labeling and Immunodetection Techniques—Subconfluent cultures of FRTL-5 or HeLa transfected cells were washed twice in phosphate-free DMEM, then labeled in phosphate-free medium containing 0.5 mCi of [^{32}P]orthophosphate/ml for 3–4 h. Cells were then lysed in a buffer containing 10 mM sodium phosphate (pH 7.4), 0.1 M NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, and 5 mM sodium vanadate. Extracts were clarified by centrifugation, an excess of TTF-1 antibody (6) was added, and immune complexes were recovered on protein A-Sepharose beads (Pharmacia Biotech Inc.). Blocking experiments were performed by preincubation of the anti-peptides antibody with an excess of the corresponding synthetic antigenic peptide. Bound proteins were boiled in SDS-PAGE sample buffer and resolved by electrophoresis in SDS-10% polyacrylamide gels (SDS-10% PAGE). Labeling of HeLa cells was performed in a similar manner. The cells were transfected with 15 μg of the expression vectors carrying either wild-type TTF-1 or the deletion and point mutants, and after 36 h the cells were labeled and immunoprecipitated as above. For pulse-chase experiments, FRTL-5 or transfected HeLa cells were labeled in methionine-free DMEM containing 0.5 mCi of [^{35}S]methionine for 1 h (pulse), the medium was then removed, and the cells were incubated in DMEM containing an 100-fold excess of nonradioactive methionine (chase). For Western blot analysis, the samples were electroblotted onto Immobilon membrane after SDS-PAGE, the blots were probed with the same TTF-1 antibody used in immunoprecipitation, and the immunocomplexes were identified by ECL (Amersham Corp.).

Phosphoamino Acids Analysis and Two-dimensional Phosphopeptide Mapping—The immunoprecipitated proteins were resolved on SDS-PAGE and subjected to a brief autoradiography, and the ^{32}P-labeled TTF-1 band was excised and eluted from the acrylamide. Phosphoamino acid analysis and phosphopeptide mapping by two-dimensional separation on thin layer cellulose plates were carried out as described elsewhere (20).

DNA-binding Assay—For gel shift assays, binding reactions were carried out by incubating the proteins for 45 min in binding buffer (20 mM Tris, pH 7.6, 75 mM KCl, 100 mM NaCl, 3 mM dithiothreitol, 10% glycerol, 1 mg/ml bovine serum albumin, 30 μg/ml poly(dI·dC) in a 20-μl final volume) containing increasing concentrations of labeled oligonucleotide C. Bound and free DNA were visualized by autoradiography, and the data obtained from each titration were plotted in graphs. The following equilibrium equation was fitted to the data by nonlinear least-squares in order to calculate K_D: [bound] = $P_o \cdot K_D^{-1} \cdot$[free]/(1 + $K_D^{-1} \cdot$[free]), where P_o is the total concentration of TTF-1, K_D is the dissociation constant, and [bound] and [free] are the concentrations of the bound and free oligonucleotide C probe, respectively.

In Vitro Kinase Assay—Purified TTF-1 from *E. coli* was used for *in vitro* labeling with protein kinase C. Phosphorylation conditions were the following. The reaction was carried out in 20 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 20 μM phospholipids (phosphatidylserine and diolein), 10 μCi of [γ-^{32}P]ATP, and 10 microunits of the kinase in each sample. Protein kinase C from rat brain was purchased from Boehringer Mannheim. After labeling, the samples were resolved by SDS-PAGE.

RESULTS

TTF-1 Is Phosphorylated on Serine Residues in Three Tryptic Peptides—FRTL-5 cells and HeLa cells, transiently transfected with a TTF-1 expression vector, were metabolically labeled with [^{32}P]orthophosphate and TTF-1 was immunoprecipitated from whole cells extracts by means of a specific anti-peptide antibody. After electrophoresis and autoradiography, TTF-1 was detected as a band of the expected molecular mass (~43 kDa) (Fig. 1, *Panel A*, *lanes 1* and *3*). The specificity of the immunoprecipitate was demonstrated by competition experiments performed in the presence of an excess of the TTF-1 peptide used for immunization (*lanes 2* and *4*). When TTF-1 was revealed by Western blot in extracts from FRTL-5 and transfected HeLa cells, a similar TTF-1 doublet was detected in both cell lines (Fig. 1, *Panel A*, *lanes 5* and *6*). To determine the amino acid residue(s) involved in phosphorylation, immunoprecipitated, ^{32}P-labeled TTF-1 from both FRTL-5 and transfected HeLa cells was acid-hydrolyzed and subjected to thin layer electrophoresis/chromatography. In both cell lines, the result indicated that TTF-1 is phosphorylated exclusively on serine residues (Fig. 1, *Panel B*). Furthermore, the labeled TTF-1 band, eluted from the acrylamide gel after immunoprecipitation, was extensively digested with trypsin, and the resulting phosphopeptides were resolved by two-dimensional electrophoresis/chromatography (Fig. 1, *Panel C*). Three similar phos-

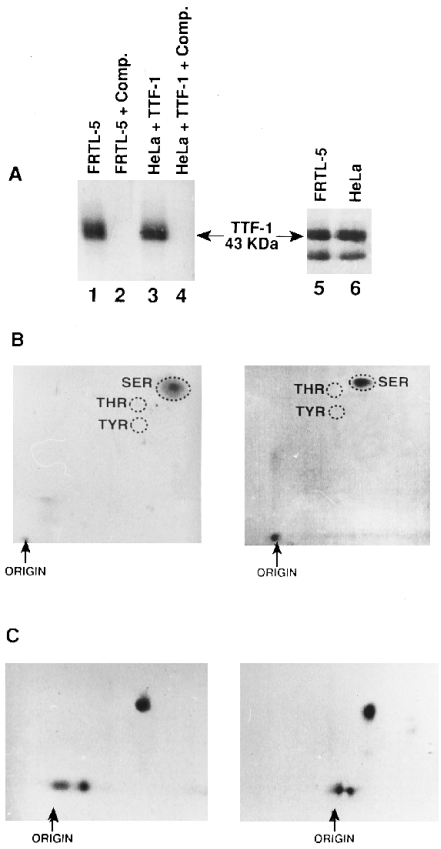

FIG. 1. **TTF-1 is equally phosphorylated in FRTL-5 cells and upon expression in HeLa cells.** *Panel A*, immunoprecipitation of TTF-1 from FRTL-5- and HeLa-transfected cells. *Left*, either thyroid cells (*FRTL-5*) or HeLa cells transfected with a TTF-1 expression vector were labeled with [^{32}P]orthophosphate. Equal amounts of TTF-1 were immunoprecipitated from the cell lysates with specific antibodies, the protein was resolved by a 8% SDS-PAGE and detected by autoradiography as described under "Materials and Methods." *Lanes 1* and *3* show the phosphorylated TTF-1 from both cell types. The specificity of the immunoprecipitation is demonstrated by competition with an excess of the antigenic peptide (*lanes 2* and *4*). *Lanes 5* and *6* show a Western blot of TTF-1 from FRTL-5 and transfected HeLa cells, respectively. *Panel B*, phosphoamino acid analysis of TTF-1 immunoprecipitated from FRTL-5 and transfected HeLa cells (*left* and *right*, respectively). Following detection by autoradiography (see *Panel A*) the bands corresponding to ^{32}P-TTF-1 from FRTL-5 and Hela cells were cut from the gel, and the protein was processed as described under "Materials and Methods." The plates were exposed for autoradiography at −70 °C for 14 days with an intensifying screen. *Panel C*, tryptic maps of TTF-1 from FRTL-5- and HeLa-transfected cells (*left* and *right*, respectively). ^{32}P-TTF-1 was purified by immunoprecipitation and electrophoresis, then eluted from the gel and subjected to tryptic digestion as described under "Materials and Methods." Tryptic digests (~500 cpm/each) were applied to thin layer cellulose plates, then resolved in the horizontal dimension by electrophoresis at pH 8.9 (anode to the *left*) and the vertical dimension by ascending chromatography as described under "Materials and Methods." The plates were exposed for autoradiography at −70 °C for 10 days with an intensifying screen. The *arrow* marks the site of sample application.

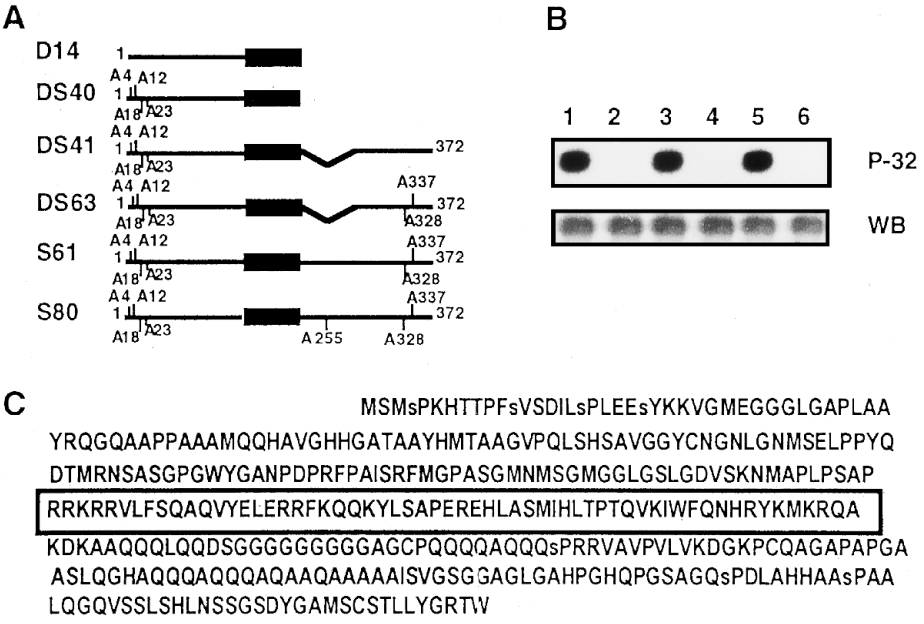

FIG. 2. **Identification of phosphorylated serine residues in TTF-1.** *Panel A*, schematic representation of TTF-1 mutants carrying the Ser/Ala substitutions. The constructs were generated as described under "Materials and Methods." *Panel B*, HeLa cells transfected with expression vectors encoding the mutated proteins were *in vivo* labeled with ^{32}P, and TTF-1 was purified by immunoprecipitation, then subjected to electrophoresis, transferred to nitrocellulose, and subjected to autoradiography (*P-32*) or Western blot (*WB*) for quantification. *Lanes 1, 2, 3, 4, 5,* and *6* are respectively *D14, DS40, DS41, DS63, S61,* and *S80*. *Panel C*, the amino acid sequence of TTF-1 showing the major phosphorylation sites. The seven identified phosphoserines are indicated in *lower case letters*. The homeodomain is *boxed*.

phopeptides could be detected (numbered 1, 2, and 3) whose identity in the two cell lines used was confirmed by mixing experiments (data not shown). These results validate the use of HeLa cells as an heterologous expression system for the study of TTF-1 phosphorylation.

Mapping of TTF-1 Phosphorylation Sites—In order to map the phosphorylated serine residues in TTF-1, a series of deletion and point mutation constructs were generated. Preliminary experiments using deletion mutants allowed us to localize the phosphorylated residues in two regions, one near the amino terminus of TTF-1 and the second on the carboxyl-terminal side of the homeodomain (data not shown). We then focused on these regions and mutagenized the codons for those serine residues that, on the basis of their sequence context, were candidate phosphorylation sites, to alanine codons (Fig. 2, *Panel A*). The resulting mutant proteins were expressed in HeLa cells, metabolically labeled with [^{32}P]orthophosphate, immunoprecipitated from whole cell extracts, and analyzed by SDS-PAGE and autoradiography to examine their level of phosphorylation (Fig. 2*B*, *P-32*). The presence of comparable levels of TTF-1 in the different transfections was monitored by Western blot (Fig. 2*B*, *WB*). A schematic drawing of the most relevant mutants and their phosphorylation level is shown in Fig. 2, *Panels A* and *B*, respectively. Deletion mutant *D14*, that lacks all the region on the carboxyl-terminal side of TTF-1 homeodomain, is phosphorylated, but replacement of serines 4, 12, 18, and 23 with alanines (mutant *DS40*) results in a complete loss of phosphorylation, indicating that one or more of these serines are the substrate for protein kinases. Mutant *DS41* encodes for a TTF-1 mutated at the amino terminus as DS40 and deleted of residues 221–294 at the carboxyl terminus of the protein. Hence, DS41 phosphorylation must be located among residues 295–372. Mutations of codons for serines 328 and 337 within this region results in a loss of phosphorylation, thus allowing the identification of these two additional phosphorylation sites. Mutant *S61* encodes a full-length TTF-1 protein containing Ser to Ala substitutions at residues 4, 12, 18, 23, 328, and 337. (Hence S61 cannot be phosphorylated at neither of the two previously identified sites.) Since S61 is phosphorylated, additional site(s) must exist among residues 221–294, in the region immediately downstream the homeodomain. Substitution of serine 255 with an alanine residue generates a full-length protein, named *S80*, completely unable to incorporate inorganic ^{32}P. A summary of all the mapped phosphoserines is shown in Fig. 2, *Panel C*. From this mutagenesis study we can conclude that the identified 7 serines are the major phosphorylation sites of TTF-1.

The Overall DNA Binding Activity of TTF-1 Is Phosphorylation-independent—Even though none of the mapped phosphorylation sites lies in the DNA-binding domain of TTF-1, we asked whether the ability of TTF-1 to bind to its target sequence could depend upon phosphorylation. To test this hypothesis, purified TTF-1 made in HeLa cells (Fig. 3, *Panel A*), purified TTF-1 produced in *E. coli* (Fig. 3, *Panel B*), total extracts of HeLa cells expressing either wild-type TTF-1 or mutant S80 (Fig. 3, *Panels C* and *D*, respectively), and FRTL-5 nuclear extract (Fig. 3, *Panel E*) were compared for their affinity toward the oligonucleotide C, containing a high affinity TTF-1 binding site (17). The amount of TTF-1 from different sources has been previously normalized by silver staining for the purified proteins and by Western blot for the extracts (data not shown). From the data shown in Fig. 3 it is evident that none of the Ala to Ser replacements has an effect on TTF-1 binding activity (Fig. 3, *Panels C* and *D*). Furthermore, bacterial TTF-1 shows the same affinity toward the C oligonucleotide as the protein purified from HeLa cells or that present in FRTL-5 nuclear extract (Fig. 3, *Panels A, B,* and *E*). Taken together these experiments suggest that phosphorylation does not directly influence the ability of TTF-1 to bind DNA.

Nonphosphorylated TTF-1 Can Activate Transcription in HeLa Cells—We next approached the question of whether phosphorylation modulates the ability of TTF-1 to activate transcription. To this end, several expression vectors encoding either wild-type or mutated TTF-1 and a reporter gene under the control of the Tg promoter were co-transfected in HeLa cells. As shown in Fig. 4, all TTF-1 mutants were able to activate transcription as well as the wild-type protein. We conclude that in heterologous HeLa cells transient transfection assay TTF-1 does not require phosphorylation to activate transcription.

TTF-1 Protein Exhibits a Slow Turnover in Both FRTL-5 and HeLa Cells—The turnover of TTF-1 was studied in a pulse-chase experiments. As shown in Fig. 5, the amount of ^{35}S-labeled TTF-1 decreased by 50% in about 14 h in FRTL-5 cells. In addition, HeLa cells were transfected with an equal amount of wild-type TTF-1 or mutant S80 which has all the seven

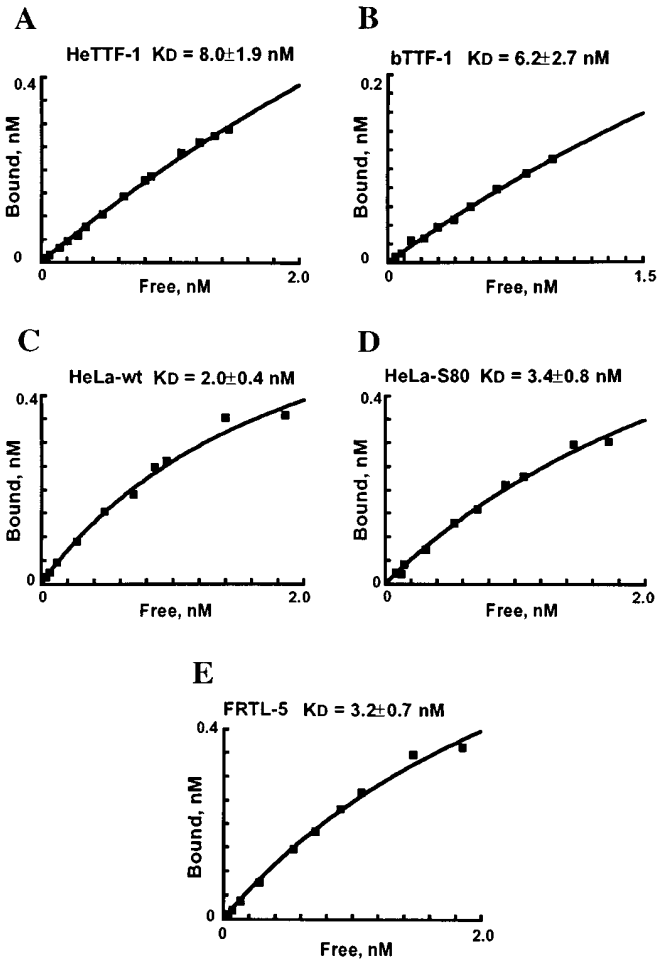

FIG. 3. **Phosphorylation does not affect the affinity of TTF-1 for the oligonucleotide C.** Purified HeTTF-1 (0.5 ng) (*Panel A*), purified bTTF-1 (2 ng) (*Panel B*), HeLa-transfected total extracts (about 3 μg) containing 0.5 ng of wild-type TTF-1 (*Panel C*), or 0.5 ng of mutant S80 (*Panel D*), or FRTL-5 nuclear extracts (about 2 μg) containing 0.5 ng of TTF-1 (*Panel E*) (the concentrations of TTF-1 in the extracts were determined by Western blotting analysis) were incubated for 45 min in binding buffer containing increasing concentrations of labeled oligonucleotide C. Bound and free DNA were visualized by autoradiography, and the data obtained from each titration were plotted in the *graphs A–E*. HeTTF-1:TTF-1 was purified from overexpressing HeLa cells (37) and bTTF-1:TTF-1 was purified from overexpressing *E. coli* cells.

mapped serines substituted with alanines. The half-life of wild-type TTF-1 in HeLa cells is also 14 h and no significant alteration was observed with the S80 mutant (data not shown).

Protein Kinase C Phosphorylates TTF-1 in Vitro—In order to determine whether any of the kinases for which a consensus sequence is present among the phosphorylation sites of TTF-1 were indeed capable of phosphorylating this transcription factor, we performed *in vitro* phosphorylation experiments. Among several protein kinases tested, only protein kinase C was able to phosphorylate TTF-1 *in vitro* (Fig. 6) (data not shown). These data suggest that TTF-1 could be a target for a specific cell signaling pathway.

DISCUSSION

In this study, we have identified the phosphorylation sites of TTF-1, a transcription factor implicated in the activation of both thyroid- (21, 22) and lung-specific (8, 9) transcriptional units. TTF-1 is phosphorylated exclusively on serine residues in three different tryptic peptides. The number of phosphorylated serines may range from a minimum of 5 to a maximum of 7 sites, given the uncertainty on the cluster of serines at the amino terminus. Fine mapping of the sites has been obtained in HeLa cells, by measuring the incorporation of labeled phosphate into TTF-1 mutants missing putative phosphorylation sites. Interestingly, no sites for protein kinase A are observed, even though an important role has been proposed for phosphorylation of TTF-1 by this protein kinase (15). The sites that we have identified show homology to CKII (Ser-18), protein kinase C (Ser-4, -23, and -255), or microtubule-associated protein kinase (Ser-328 and -337) phosphorylation sites. These protein kinases are components of signal transduction pathways that have been shown to control thyroid function and to be activated in thyroid cells in response to a variety of stimuli (23). In our *in vitro* experiments only protein kinase C was able to phosphorylate TTF-1. This result is of interest since activation of protein kinase C has been suggested to inhibit thyroid cell differentiation (24–30). Furthermore, activation of protein kinase C has been implicated in TSH stimulation of thyroid cell growth (31, 32). Since TTF-1 has been implicated both in the expression of thyroid differentiated function (21) and in the control of thyroid cell growth (33), it is an attractive hypothesis that some of these functions could be controlled via phosphorylation of TTF-1 by protein kinase C. The data presented in this study do not, at present, support this hypothesis, since we could not demonstrate any alteration in TTF-1 activity as a consequence of the lack of phosphorylation. However, we cannot rule out the possibility that we were unable to provide evidence for the relevance of phosphorylation in TTF-1 because of the transient transfection assay in heterologous cells that we used. The regulation of TTF-1 through phosphorylation may impinge on thyroid-specific mechanisms which do not operate in HeLa cells such as, for example, phosphorylation-dependent interaction (34) with specific co-activators. Future experiments should aim at studying the phosphorylation mutants in thyroid cells. More conclusive are our studies on the role of phosphorylation in the DNA binding activity of TTF-1. Phosphorylation has been indicated as a critical step for the binding of TTF-1 to its target sequence on the Tg promoter (15, 35) as well as on the TSHr one (36). Moreover, TTF-1 binding was shown to be abrogated when nuclear extracts were incubated with acid phosphatases. Treatment of extracts with protein kinase A (15) was able to restore TTF-1 DNA binding activity, leading to the conclusion that TTF-1 is directly modified by protein kinase A and binds to the Tg promoter only if phosphorylated (35). In contrast, our results clearly show that phosphorylation does not play an important role in the overall TTF-1 DNA binding activity. This conclusion is based on the comparable affinities toward a double-stranded oligonucleotide containing a well characterized TTF-1 binding site (oligonucleotide C) (17) of wild-type TTF-1, purified from either bacteria or animal cells, and of the S80 mutant, that contains less that 0.1 phosphorus atom/molecule. We cannot exclude that ancillary proteins, which are protein kinase A substrates, could either help or interfere with TTF-1 binding (15), depending on the physiological conditions. In addition, we have recently discovered that TTF-1 DNA binding activity can be easily lost during extract preparation and can be readily recovered by exposure to reducing agents (37). It is also conceivable that the exquisite sensitivity of TTF-1 to oxidation could interfere with the interpretation of *in vitro* treatments with phosphatases and kinases.

Previous data from our laboratory have demonstrated that TTF-1 is inactive in Ha-^{ras}-transformed cells, although it is present and capable of binding to DNA (14). In this cell line, we have observed a reduced phosphorylation of TTF-1 (14), and we proposed that this could be the cause for the inactivity of TTF-1 in transformed cells. In this respect, it is interesting to note that *ras* activation has been reported to affect the stimulation

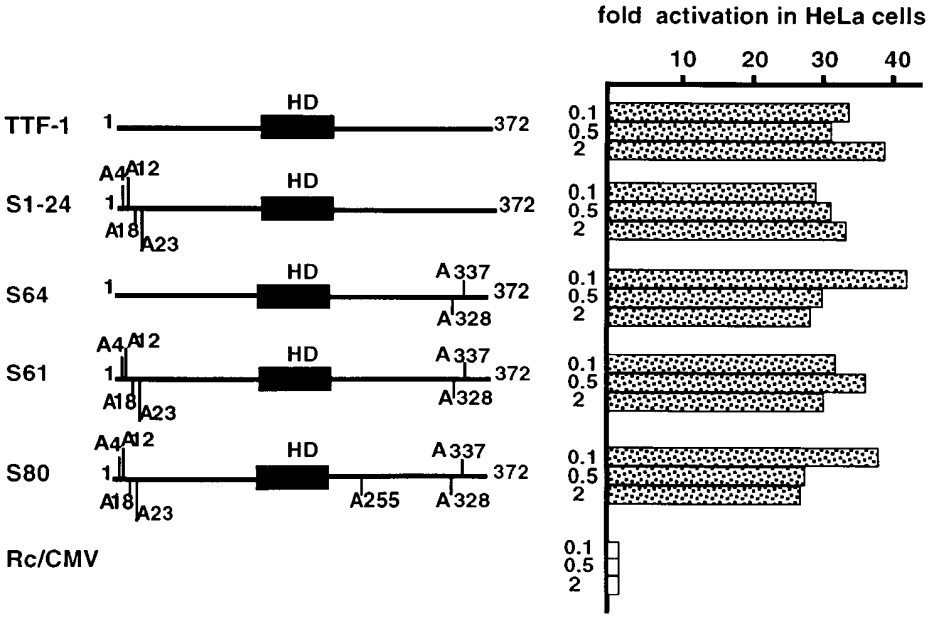

FIG. 4. **Transactivation of the thyroglobulin promoter containing TTF-1 binding sites, by wild-type TTF-1 and various mutant proteins.** The indicated amount (expressed in micrograms) of expression vectors encoding TTF-1 either wild type or mutated at the phosphorylation sites were transiently transfected into HeLa cells together with 5 μg of a reporter construct carrying the thyroglobulin promoter fused to the chloramphenicol acetyltransferase gene. The activation values were obtained by dividing the enzymatic activity present in extracts of cells transfected with the various TTF-1 proteins by the activity obtained with the empty expression vector (*Rc/CMV*).

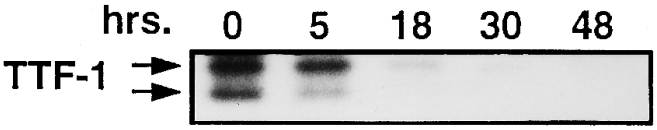

FIG. 5. **Half-life of TTF-1 protein.** FRTL-5 cells expressing endogenous TTF-1 were *in vivo* labeled with [^{35}S]methionine in a pulse-chase experiment as described under "Materials and Methods." After the labeling, the chase was performed at the different times indicated. TTF-1 protein was purified by immunoprecipitation, then subjected to SDS-PAGE and exposed to autoradiography .

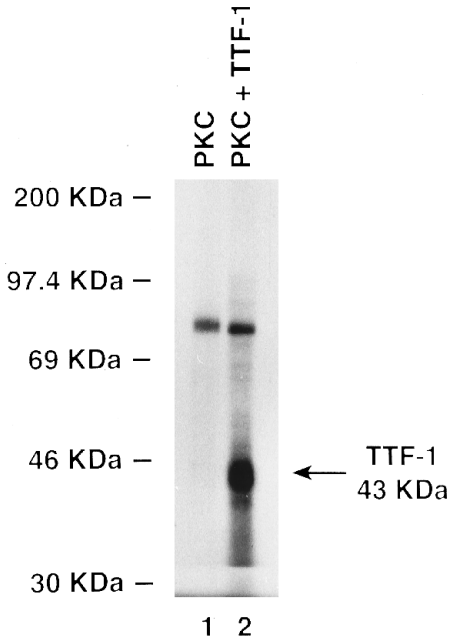

FIG. 6. **TTF-1 can be phosphorylated by protein kinase C *in vitro*.** Bacterially expressed TTF-1 protein was phosphorylated *in vitro* with protein kinase C. Samples were loaded on a 8% polyacrylamide gel, and phosphorylated proteins were visualized by autographic exposure of the dried gel. To exclude any possible effect from autophosphorylation of the kinase, the experiment was also performed in the absence of the substrate (*lane 1*).

of protein kinase C in *Xenopus* oocytes (38) and in PC12 cells (39). It is also noteworthy the role of Ras proteins in signal transduction pathways initiated in membrane receptors (insulin-like growth factor-I, insulin, and epidermal growth factor) and involving microtubule-activating protein kinases. These alterations in protein kinases activities can be somehow affecting TTF-1, either by a direct or an indirect mechanism involving other kinases and/or phosphatases. The characterization of the phosphorylation sites in TTF-1 may be instrumental for the elucidation of the mechanisms leading to the interference between transformation and differentiation in thyroid cells.

REFERENCES

1. Guazzi, S., Price, M., de Felice, M., Damante, G., Mattei, M. G., and Di Lauro, R. (1990) *EMBO J.* **9,** 3631–3639
2. Musti, A. M., Ursini, V. M., Avvedimento, E. V., Zimarino, V., and Di Lauro, R. (1987) *Nucleic Acids Res.* **15,** 8149
3. Sinclair, A. J., Lonigro, R., Civitareale, D., Ghibelli, L., and Di Lauro, R. (1990) *Eur. J. Biochem.* **193,** 311–318
4. Francis-Lang, H., Price, M., Polycarpou-Schwarz, M., and Di Lauro, R. (1992) *Mol. Cell. Biol.* **12,** 576–588
5. De Felice, M., Damante, G., Zannini, M., Francis-Lang, H., and Di Lauro, R. (1995) *J. Biol. Chem.* **270,** 26649–26656
6. Lazzaro, D., Price, M., De Felice, M., and Di Lauro, R. (1991) *Development* **113,** 1093–1104
7. Ledent, C., Parmentier, M., and Vassart, G. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 6176–6180
8. Bohinski, R. J., Di Lauro, R., and Whitsett, J. A. (1994) *Mol. Cell. Biol.* **14,** 5671–5681
9. Bruno, M. D., Bohinski, R. J., Huelsman, K. M., Whitsett, J. A., and Korfhagen, T. (1995) *J. Biol. Chem.* **270,** 6531–6536
10. Hunter, T., and Karin, M. (1992) *Cell* **70,** 375–387
11. Jackson, S. P. (1992) *Trends Cell Biol.* **2,** 104–108
12. Karin, M. (1994) *Curr. Opin. Cell Biol.* **6,** 415–424
13. Karin, M., and Hunter, T. (1995) *Curr. Biol.* **5,** 747–757
14. Francis-Lang, H., Zannini, M., De Felice, M., Berlingieri, M. T., Fusco, A., and Di Lauro, R. (1992) *Mol. Cell. Biol.* **12,** 5793–5800
15. Avvedimento, E. V., Musti, A. M., Ueffing, M., Obici, S., Gallo, A., Sanchez, M., DeBrasi, D., and Gottesman, M. E. (1991) *Genes & Dev.* **5,** 22–28
16. Ambesi-Impiombato, F. S., and Coon, H. G. (1979) *Int. Rev. Cytol.* **10,** 163–172
17. Civitareale, D., Lonigro, R., Sinclair, A. J., and Di Lauro, R. (1989) *EMBO J.* **8,** 2537–2542
18. Gorman, C. M., Moffat, L. M., and Howard, B. H. (1982) *Mol. Cell. Biol.* **2,** 1044–1051
19. deWet, J. R., Wood, K. V., Deluca, M., Helinski, D. R., and Subramani, S. (1987) *Mol. Cell. Biol.* **7,** 725–737
20. Boyle, W. J., Geer, P. v. d., and Hunter, T. (1991) *Methods Enzymol.* **201,** 110–149
21. Damante, G., and Di Lauro, R. (1994) *Biochim. Biophys. Acta* **1218,** 255–266
22. Zannini, S., De Felice, M., Sato, K., and Di Lauro, R. (1994) in *Transcription Factors and Differentiation of Thyroid Follicular Cells. Frontiers in Endocrynology* (Sizonenko, P. C., Aubert, M. L., and Vassalli, J.-D., eds) pp. 79–87, Ares Serono Symposia Publications, Geneva, Switzerland
23. Dumont, J. E., and Vassart, G. (1995) in *Endocrinology* (DeGroot, L. J., ed) Vol. 1, 3rd Ed., pp. 543–559, W. B. Saunders, Philadelphia
24. Bachrach, L. K., Eggo, M. C., Mak, W. W., and Burrow, G. N. (1985) *Endocrinology* **116,** 1603–1609
25. Ginsberg, J., and Murray, P. G. (1986) *FEBS Lett.* **206,** 309–312
26. Lombardi, A., Veneziani, B. M., Tramontano, D., and Ingbar, S. H. (1988) *Endocrinology* **123,** 1544–1552
27. Pohl, V., Roger, P. P., Christophe, D., Pattyn, G., Vassart, G., and Dumont,

J. E. (1990) *J. Cell Biol.* **111,** 663–672
28. Roger, P. P., Reuse, S., Servais, P., VanHeuverswyn, B., and Dumont, J. E. (1986) *Cancer Res.* **46,** 898–906
29. Vassart, G., and Dumont, J. E. (1992) *Endocr. Rev.* **13,** 596–611
30. Kraiem, Z., Sadeh, O., Yosef, M., and Aharon, A. (1995) *Endocrinology* **136,** 585–590
31. Fujimoto, J., and Brenner-Gati, L. (1992) *Endocrinology* **130,** 1587–1592
32. Dumont, J. E., Lamy, F., Roger, P., and Maenhaut, C. (1992) *Physiol. Rev.* **72,** 667–697
33. Rossi, D. L., Acebron, A., and Santisteban, P. (1995) *J. Biol. Chem.* **270,** 23139–23142
34. Chrivia, J. C., Kwok, R. P., Lamb, N., Hagiwara, M., Montminy, M. R., and Goodman, R. H. (1993) *Nature* **365,** 855–859
35. Gallo, A., Benusiglio, E., Bonapace, I. M., Cassano, A. F. S., Garbi, C., Musti, A. M., Gottesman, M. E., and Avvedimento, E. V. (1992) *Genes & Dev.* **6,** 1621–1630
36. Shimura, H., Okajima, F., Ikuyama, S., Shimura, Y., Kimura, S., Saji, M., and Kohn, L. D. (1994) *Mol. Endocrinol.* **8,** 1049–1069
37. Arnone, M. I., Zannini, M., and Di Lauro, R. (1995) *J. Biol. Chem.* **270,** 12048–12055
38. Lacal, J. C., Pena, P. d. l., Moscat, J., Garcia-Barreno, P., Anderson, P. S., and Aaronson, S. A. (1987) *Science* **238,** 533–536
39. Lacal, J. C., Cuadrado, A., Jones, J. E., Trotta, R., Burstein, D. E., Thomson, T., and Pellicer, A. (1991) *Mol. Cell. Biol.* **10,** 2983–2990